RayBio®Phospho-EGFR (Tyr 1086) Kit

For Measuring Phospho-EGFR (Tyr 1086) in Human Cell Lysates

User Manual (Revised Mar 1, 2012)

RayBio® Phospho-EGFR (Tyr 1086) ELISA Kit Protocol

(Cat#: PEL-EGFR-Y1086)

RayBiotech, Inc.

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I. INTRODUCTION

RayBio®Phospho-EGFR (Tyr 1086) ELISA (Enzyme-Linked Immunosorbent Assay) kit is a very rapid, convenient and sensitive assay kit that can monitor the activation or function of important biological pathways in cell lysates. By determining phosphorylated EGFR protein in your experimental model system, you can verify pathway activation in your cell lysates. You can simultaneously measure numerous different cell lysates without spending excess time and effort in performing a Western Blot analysis.

This Sandwich ELISA kit is an in vitro enzyme-linked immunosorbent assay for the measurement of human phospho-EGFR (Tyr 1086). An anti-EGFR antibody has been coated onto a 96-well plate. Samples are pipetted into the wells and phosphorylated and unphosphorylated EGFR present in a sample is bound to the wells by the immobilized antibody. The wells are washed and anti-phospho-EGFR (Tyr 1086) antibodies are used to detect phosphorylated EGFR. After washing away unbound antibody, HRP-conjugated anti-Rabbit IgG (secondary antibody) is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of EGFR bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

II. MATERIAL PROVIDED

1. EGFR Microplate (Item A): 96 wells (12 strips x 8 wells) coated with monoclonal anti-EGFR.

2. Wash Buffer Concentrate (20x) (Item B): 25 ml of 20x concentrated solution
3. Anti-phospho-EGFR (Tyr 1086) (Item C): 11μl rabbit anti-EGFR (Tyr 1086).
4. HRP-conjugated Anti-rabbit IgG (Item D-1), 25 μl of 500x concentrated HRP-conjugated Anti-rabbit IgG.
5. Assay Diluent (Item E2): 15 ml of 5x concentrated buffer. For diluting cell lysate sample and reagent (Item C and Item D-1).
6. TMB One-Step Substrate Reagent (Item H): 12 ml of 3,3',5,5'-tetramethylbenzidine (TMB) in buffered solution.
7. Stop Solution (Item I): 8 ml of 0.2 M sulfuric acid.
8. Cell Lysate Buffer (Item J): 5 ml 2x Cell Lysate Buffer (not including protease and phosphatase inhibitors).
9. Positive Control A431S001-1 (Item K): 1 vial of lyophilized powder from A431 cell lysate.

III. STORAGE

Upon receipt, the kit should be stored at –20 °C. Please use within 6 months from the date of shipment. After initial use, Wash Buffer Concentrate (Item B), HRP-conjugated Anti-rabbit IgG (Item D-1), Assay Diluent (Item E2), TMB One-Step Substrate Reagent (Item H), Stop Solution (Item I) and Cell Lysate Buffer (Item J) should be stored at 4 °C to avoid repeated freeze-thaw cycles. Anti-phospho-EGFR (Tyr 1086) (Item C) should be stored at –20 °C. Return unused wells to the pouch containing desiccant pack, reseal along entire edge and store at –20 °C. Reconstituted Positive Control (Item K) should be stored at -70 °C.

IV. ADDITIONAL MATERIALS REQUIRED

1 Microplate reader capable of measuring absorbance at 450 nm.
2 Protease and Phosphatase inhibitors.
3 Shaker.
4 Precision pipettes to deliver 2 μl to 1 ml volumes.
5 Adjustable 1-25 ml pipettes for reagent preparation.
6 100 ml and 1 liter graduated cylinders.
7 Distilled or deionized water.
8 Tubes to prepare sample dilutions.

V. SAMPLE PREPARATION

Cell lysates - Rinse cells with PBS, making sure to remove any remaining PBS before adding the Cell Lysate Buffer. Solubilize cells at 4 x 10^7 cells/ml in 1x Cell Lysate Buffer (we recommend adding protease and phosphatase inhibitors to Cell Lysate Buffer prior to sample preparation). Pipette up and down to resuspend and incubate the lysates with shaking at 2 - 8° C for 30 minutes. Microcentrifuge at 13,000 rpm for 10 minutes at 2 - 8° C, and transfer the supernates into a clean test tube. Lysates should be used immediately or aliquoted and stored at –70 °C. Avoid repeated freeze-thaw cycles. Thawed lysates should be kept on ice prior to use.

For the initial experiment, we recommend to do a serial dilution testing such as 5-fold and 100-fold dilution for your cell lysates with Assay Diluent (Item E2) before use.

Note: The fold dilution of sample used depends on the abundance of phosphorylated proteins and should be determined empiricallys. More of the sample can be used if signals are too weak. If signals are too strong, the sample can be diluted further.

Cell Lysate Buffer should be diluted 2-fold with deionized or distilled water before use (recommend to add protease and phosphatase inhibitors).

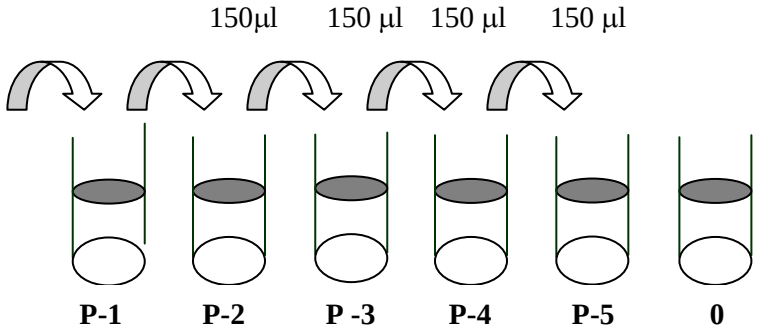
VI. REAGENT PREPARATION

1. Bring all reagents and samples to room temperature (18 - 25°C) before use.

2. Item E2, Assay Diluent should be diluted 5-fold with deionized or distilled water before use.

3. Preparation of Positive Control: Briefly spin the Positive Control vial of Item K. Add 800 µl 1x Assay Diluent (Item E2, Assay Diluent should be diluted 5-fold with deionized or distilled water before use) into Item K vial to prepare a Positive Control (P-1) Solution (See i. Positive control of part IX. TYPICAL DATA for a typical result). **Dissolve the powder thoroughly by a gentle mix** (it can be removed by centrifuge if any precipitate in the solution is found). Pipette 300 µl 1x Assay Diluent into each tube. Use the Positive Control (1) to produce a dilution series (shown below). Mix each tube thoroughly before the next transfer. 1x Assay Diluent serves as the background.

Positive Control, Item K vial
+ 800 µl 1x Assay Diluent



4. If the Wash Concentrate (20x) (Item B) contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute 20 ml of Wash Buffer Concentrate into deionized or distilled water to yield 400 ml of 1x Wash Buffer.

5. Briefly spin the anti-phospho-EGFR (Tyr 1086) (Item C) before use. Pipette up and down to mix gently. The anti-phospho-EGFR (Tyr 1086) should be diluted 1,000-fold with 1x Assay Diuent. For example, add 5 µl anti-phospho-EGFR (Tyr 1086) into a tube with 5.0 ml 1x Assay Diluent to prepare a 1,000-fold diluted antibody.

6. Briefly spin the HRP-conjugated anti-rabbit IgG (Item D-1) before use. Pipette up and down to mix gently. HRP-conjugated anti-rabbit IgG concentrate should be diluted 500-fold with 1x Assay Diuent.

   For example: Briefly spin the vial (Item D-1) and pipette up and down to mix gently. Add 10 µl of HRP-conjugated anti-rabbit IgG concentrate into a tube with 5.0 ml 1x Assay Diluent to prepare a 500-fold diluted HRP-conjugated

anti-rabbit IgG solution.

7. Cell Lysate Buffer should be diluted 2-folds with deionized or distilled water before use (recommend to add protease and phosphatase inhibitors).

VII. ASSAY PROCEDURE:

1. Bring all reagents to room temperature (18 - 25°C) before use. It is recommended that all samples or Positive Control should be run at least in duplicate.

2. Add 100 µl of each sample or positive control into appropriate wells. Cover well with plate holder and incubate for 2.5 hours at room temperature or over night at 4°C with shaking.

3. Discard the solution and wash 4 times with 1x Wash Solution. Wash by filling each well with Wash Buffer (300 µl) using a multi-channel Pipette or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

4. Add 100 µl of prepared 1,000 fold diluted anti-phospho-EGFR (Tyr 1086) (Reagent Preparation step 6) to each well. Incubate for 1.5 hour at room temperature with shaking.

5. Discard Discard the solution. Repeat the wash as in step 3.

6. Add 100 µl of prepared 500 fold diluted HRP-conjugated anti-rabbit IgG (see Reagent Preparation step 7) to each well. Incubate for 1 hour at room temperature with shaking.

7. Discard Discard the solution. Repeat the wash as in step 3.

8. Add 100 µl of TMB One-Step Substrate Reagent (Item H) to each well. Incubate for 30 minutes at room temperature in the dark with shaking.

9. Add 50 µl of Stop Solution (Item I) to each well. Read at 450 nm immediately.

VIII. ASSAY PROCEDURE SUMMARY

1. Prepare all reagents, samples and standards as instructed.

⇩

2. Add 100 µl sample or positive control to each well. Incubate 2.5 hours at room temperature or over night at 4°C.

⇩

3. Add 100 µl prepared primary antibody to each well. Incubate 1.5 hours at room temperature.

⇩

4. Add 100 µl prepared secondary antibody solution. Incubate 1 hour at room temperature.

⇩

5. Add 100 µl TMB One-Step Substrate Reagent to each well.
   Incubate 30 minutes at room temperature.

⇩

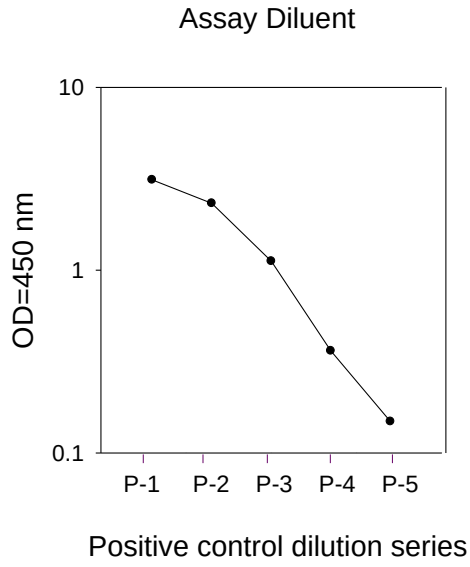
6. Add 50 µl Stop Solution to each well.
   Read at 450 nm immediately.

IX. TYPICAL DATA

ELISA data analysis: Average the duplicate readings for each sample or positive control then subtract the average blank optical density.

i. Positive Control

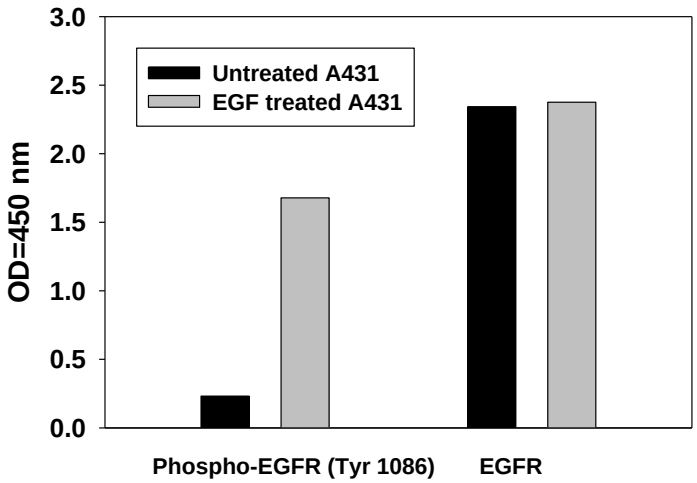
A431 cells were treated with recombinant human EGF at 37°C for 20 min. Solubilize cells at 4 x 10^7 cells/ml in Cell Lysate Buffer. Serial dilutions of lysates were analyzed in this ELISA. Please see step 3 of Part VI Reagent Preparation for detail.



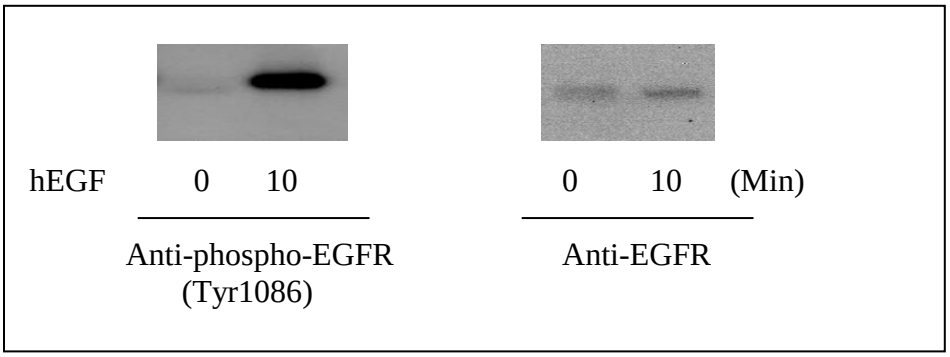
ii. Recombinant Human EGF Stimulation of A431 Cell Lines

A431 cells were treated or untreated with 100 ng/ml recombinant human EGF for 10 min. Cell lysates were analyzed using this phosphoELISA and Western Blot.

ELISA



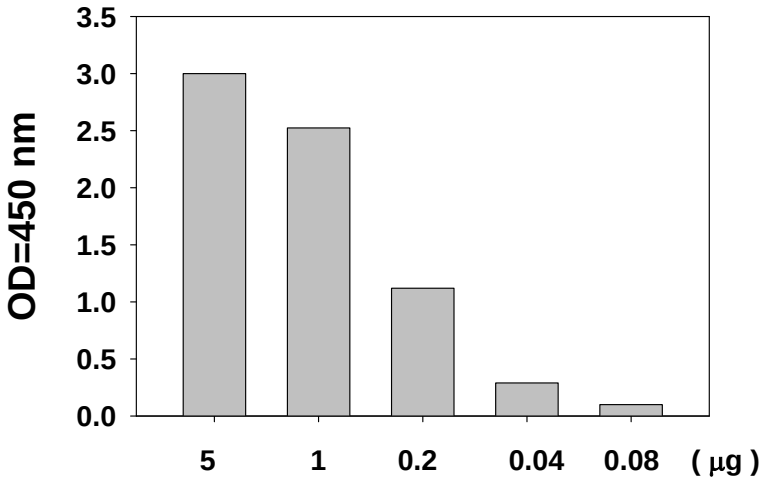
Western-Blot



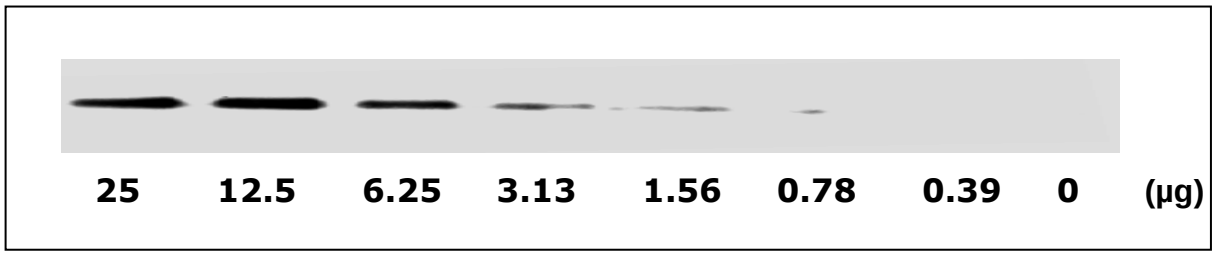
iii. SENSITIVITY

The A431 cells were treated with 100 ng/mL recombinant human EGF for 20 minutes to induce phosphorylation of EGF R. Serial dilutions of lysates were analyzed in this ELISA and by Western blot. Immunoblots were incubated with anti-phospho-EGFR (Tyr 1086).

ELISA



Western-Blot



X. REFERENCES:

1. Hackel, P.O. et al. (1999) Curr. Opin. Cell Biol. 11, 184-189.
2. Alroy, I. and Y. Yarden (1997) FEBS Lett. **410**:83.
3. Cooper, J.A. and Howell, B. (1993) Cell 73, 1051-1054.
4. Riedemann, J. et al. (2007) Biochem. Biophys. Res. Commun. **355**:707.

XI. TROUBLESHOOTING GUIDE

Problem	Cause	Solution
1. Sample signals:		
a. Too low	a. Sample concentration is too low	a. Increasing sample concentration
b. Too high	b. Sample concentration is too high	b. Reducing sample concentration
2. Large CV	a. Inaccurate pipetting	a. Check pipettes
3. High background	a. Plate is insufficiently washed	a. Review the manual for proper washing. If using an automated plate washer, check that all ports are unobstructed.
	b. Contaminated wash buffer	b. Make fresh wash buffer
4. Positive Control: Low signal	a. Improper storage of the ELISA kit	a. Upon receipt, the kit should be stored at –20 ^{o}C. Store the positive control at -70^{o}C after reconstitution.
	b. Stop solution	b. Stop solution should be added to each well before measurement and read OD immediately.
	c. Improper primary or secondary antibody dilution	c. Ensure correct dilution

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Label based antibody array: Simultaneous detection more than 500 proteins in one experiment

Quantibody Array: Quantitative measurement of multiple protein levels

Protein Array

ELISA

Cell-Based Phosphorylation ELISA

Tissue MicroArray

Protein: Cytokine, Chemokine, Adiplokine, Angiogenic factor, Virus, bacteria and infectious disease protein, hormone, Enzyme, other

Peptide

Antibody: Cytokine, Adipokine, Angiogenic factor, Signal transduction, Transcription factor, Receptor, Adhesion molecule, Virus, bacteria and other infectious agents, Secondary antibody, Tag antibody, Immunoglobulin, Hormone, Cell surface, Protease, other

Antibody array, Protein array, Peptide array, ELISA, Phosphorylation assay Tissue array

Assay service: just simply send your samples and get data in 1 to 2 weeks.

Antibody array, Protein array, ELISA, Quantibody array

Antibody production: highest quality with very competitive price

Monoclonal antibody, Recombinant antibody, Polyclonal antibody, Phase display, Antibody angineering, Antibody conjugation

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